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A Continuous Spectrophotometric Assay for the Determination of Diamondback Moth Esterase Activity

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Conventional methods to determine esterase activity from insects are composed of a three-step process where the enzyme is allowed to hydrolyze a 1-naphthyl acetate substrate, that reaction is quenched by a SDS detergent, and then a Fast Blue B dye complex is formed with 1-naphthol, the product of 1-naphthyl acetate hydrolysis. These methods measure dye-product complex rather than the product, 1-naphthol. A new assay is presented that continuously monitors the formation of 1-naphthol with the hydrolysis of an esterase substrate. The esterase activity was determined as the slope of the linear regression change in absorbance over time at 320 nm. The continuous assay provides a simple, rapid, and sensitive method for measuring esterases extracted from a single diamondback moth in 1–10 min. The detection limit of the assay is approximately 0.6 μM 1-naphthol. The 1-naphthol product from the esterase reaction was confirmed by HPLC analysis. According to the assay, the K_m and V_{max} values of the esterase were 28 ± 2 μM and 6.0 ± 0.1 μM/min, respectively, at 37°C for 1-naphthyl acetate. The K_i value was 9 ± 2 μM using azadirachtin, an insecticide from neem tree, *Azadirachta indica* (A.Juss). Azadirachtin was a reversible competitive inhibitor of the esterase activity. Arch Insect Biochem Physiol 54:68–76, 2003. Published 2003 Wiley-Liss, Inc.†

KEYWORDS: esterase; spectrophotometric assay; naphthyl acetate; naphthol; detoxification enzyme

INTRODUCTION

The diamondback moth, *Plutella xylostella* (L.), is a serious pest of cruciferous vegetables worldwide. The management of the diamondback moth has become increasingly difficult due to the development of insecticide resistance (Talekar and Shelton, 1993). Metabolic detoxification of insecticides by enzymes is a principal mechanism that contributes to the development of insecticide resistance (Terriere, 1984). Esterases belong to a large and diverse family of detoxifying enzymes (Dowd et al., 1983). Esterases protect the target-site molecule by either reducing the toxicity of the toxicant by hydrolytic activity, and/or sequestering the toxicant by competitive binding with the toxicant (Ishaaya, 1993). Esterases have a wide range of substrate specificity. They are able to cleave or hydrolyze organic functional groups such as esters, triester phosphates, halides, thioesters, amide, and peptides (Dauterman, 1982–83).

Esterase activity is traditionally measured with a multi-step assay (the endpoint assay). Initially, esterases, in a suitable buffer such as a sodium phosphate, are allowed to hydrolyze an esterase substrate such as 1-naphthyl acetate (1NA) at 27–37°C for 30–60 min (Gomori, 1953; van Asperen, 1962). The hydrolytic reaction is then quenched by the addition of a detergent solution such as sodium dodecyl sulfate (SDS) and a coupling agent

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such as Fast Blue B dye. The dye-product complex remains soluble in the presence of the SDS detergent. Absorbance of the Fast Blue B-1-naphthol (1N-OH) complex is measured at a visible range from 550 to 610 nm and the esterase activity is determined from a 1-naphthol standard curve (Dary et al., 1990). This assay has the disadvantage of measuring only the Fast Blue B-1-naphthol complex but not the direct product of hydrolysis of 1-naphthyl acetate: 1-naphthol. This procedure may lead to inaccuracy when more chemicals such as a detergent and a dye are added and the chemical conditions are changed, especially when the enzyme activity is weak. For example, neither SDS detergent nor Fast Blue RR dye completely inhibits esterase activity in mosquitoes (Grant et al., 1989). The incomplete inhibition can cause the instability of the absorbance signals of the dye-naphthol complex. Grant et al. (1989) demonstrated that esterase activity toward 1NA could be measured kinetically in microplates without SDS. In addition, diazonium salts (dyes) are pH-dependent and are used in a narrow pH range as the coupling agents are unstable in other pH regions (Johnston and Ashford, 1980). Furthermore, only two absorbance values (an initial and a randomly chosen final reading in many cases) are used to determine the initial velocity and enzyme activity in the endpoint assay. This is a serious problem for quantification since the initial velocity may not be linear during the entire time of the assay.

A new assay presented in this study measures 1-naphthol as it is produced and is extremely sensitive. Esterase activity can be determined with low concentrations of enzyme. It allows for determination of kinetic parameters including the K_m, V_{max}, and K_i. The product of 1-naphthyl acetate hydrolysis, 1-naphthol, is directly and continuously monitored at 320 nm without adding a detergent to quench the reaction and a coupling agent to measure the formation of the dye-product complex. As a result, the initial velocity (V_o) of the enzyme activity can be determined as a function of the slope change in absorbance (optical density) over a unit of time in one to ten minutes, rather than the 30 to 60 min that the dye-product complex assay requires. The continuous assay was used in this report to determine the esterase activity in extracts of the diamondback moth.

MATERIALS AND METHODS

Chemicals

1-Naphthyl acetate, 1-naphthol, dithiothreitol, Triton X-100, Nonidet P-40 and azadirachtin were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) and the Bradford dye reagent were obtained from Bio-Rad Laboratories (Richmond, CA).

Enzyme Preparations

Diamondback moth male adults were randomly selected and chilled at –20°C for 5 min. Forty diamondback moths were homogenized in 1 ml of homogenized solution (0.1 M sodium phosphate, pH 7.0, containing 0.1% Triton X-100, 35 mM sucrose, and 5 mM dithiothreitol [DTT]) using a Teflon pestle and a microcentrifuge tube at 4°C (Anspaugh et al., 1994). The homogenate was centrifuged for 30 min at 12,000*g* and 4°C by a 5417R Eppendorf Centrifuge (Westbury, NY). The supernatant was collected, aliquoted into plastic microcentrifuge tubes and stored at –80°C. Triton X-100 improved the extraction of esterases in solution (Dary et al., 1990). The extracted enzymes can be stored in the freezer with no change in esterase activity up to several months (Maa and Chuang, 1983).

Enzyme Assays

Esterase activity was measured using 1NA as a substrate. Production of 1-naphthol was monitored with a double beam Perkin-Elmer Lambda 25 UV/Vis spectrophotometer (Shelton, CT) using both a sample and a reference quartz cuvettes (1 ml) with path length of 1 cm. All assays were done in triplicate at 37°C. The reaction mixture (1 ml) contained 50 mM sodium phosphate (pH 7.0), 10 mM $MgCl_2$, 0.1% Nonidet P-40, 50 µM substrate, and 40 µg esterase protein. The enzyme blank refer-

ence cuvette was used without the protein as a control. The K_i value was measured in the presence of 0.7 μM Azadirachtin. Absorbance measurements at 320 nm were made at 0.5-sec intervals for 10 min. Absorbance values were plotted against time (minutes). Initial velocities were determined from the linear slope of progress curves (time courses) acquired using the Perkin-Elmer Enzyme-Kinetic (KinLab) software. V_o, expressed in μM/min with the concentration of 1-naphthol produced/min extrapolated from a 1-naphthol standard curve ranging from 0 to 10 μM, was derived from the linear portion of the activity curve using a molar extinction coefficient (ε) of 2,222 M^{-1} cm^{-1} (Rudnicka and Kochman, 1984). The relative esterase activities (%) are the ratios of the initial velocities with different concentrations of salts such K^+, Na^+, and Mg^{2+} and without salt. The temperature control was provided to the cuvette compartments by a Lauda Brinkmann Ecoline RE106 low-temperature thermostat (Westbury, NY). The K_m, V_{max}, and K_i values were measured using the SigmaPlot (7.101) Enzymatic Kinetics Module 1.0 software (SPSS Inc.).

Protein Determination

The protein concentration of enzyme extracts was determined by the method of Bradford (1976). Absorbance at 595 nm was compared to a protein blank. Each sample was replicated three times and the protein concentration extrapolated from a standard curve using bovine serum albumin.

HPLC Detection of 1-Naphthol

Detection of 1-naphthol was accomplished at 320 nm with a Spherisorb C8 reverse phase column, 250 mm by 4.6 mm I.D. (HEMA-RP, C8, 10 μm, Alltech, Deerfield, IL) using two Dionex HPLC Model GP 40 gradient pumps and an AD 20 absorbance detector (Sunnyvale, CA). The column was operated at ambient temperature (approximately 25°C). Mobile phase A consisted of 0.1 M ammonium acetate and mobile phase B, a 4:1 mixture of acetonitrile:water at pH 7.5. Prior to using, the mobile phases were passed through a 0.20-μm nylon member filter. Each pump delivered the mobile phase at the flow rate of 1.0 ml per minute. Absorbance at 320 nm was observed using 1-naphthol products.

RESULTS AND DISCUSSION

Continuous Spectrophotometric Assay and Confirmation of 1-Naphthol by HPLC

The amount of the product, 1-naphthol formed at 320 nm, increased with time (Fig. 1). The reaction proceeded linearly for 20 min. V_o, expressed in μM/min, was determined from the increase in A_{320} over the first 10 min. After 70 min, the reaction plateaued. There was no reaction in the absence of enzyme indicated by the zero slope (or the zero initial velocity). The initial velocity was linearly dependent on the concentrations of esterase protein in diamondback moths (Fig. 2).

1-Naphthol can be directly identified by its retention time (13.4 min.) with HPLC at 320 nm (Fig. 3). However, 1-naphthyl acetate substrate cannot be measured at the same condition because of minimal absorbance. The degree of hydrolysis of 1-naphthyl acetate under the reaction of esterase was shown as a weak residual peak without the enzyme and a strong peak (> 8-fold) with the enzyme. The small peak in the control is due to 1-naphthol contamination in the acetate or represents spontaneous degradation. The results from HPLC confirmed the findings from the new assay.

The continuous method is to accurately determine the kinetic linearity for each individual reaction because the double-beam technique is used that automatically records the net absorbance signal differences between a sample and a control during the enzymatic reaction. Therefore, it will limit the background errors from chemical compounds including 1-naphthol from the autohydrolysis of 1-naphthyl acetate. Moreover, the accuracy of the reaction rate determinations for an individual sample is greater with the continuous assay than the endpoint methods because the absorbance is determined at multiple time points.

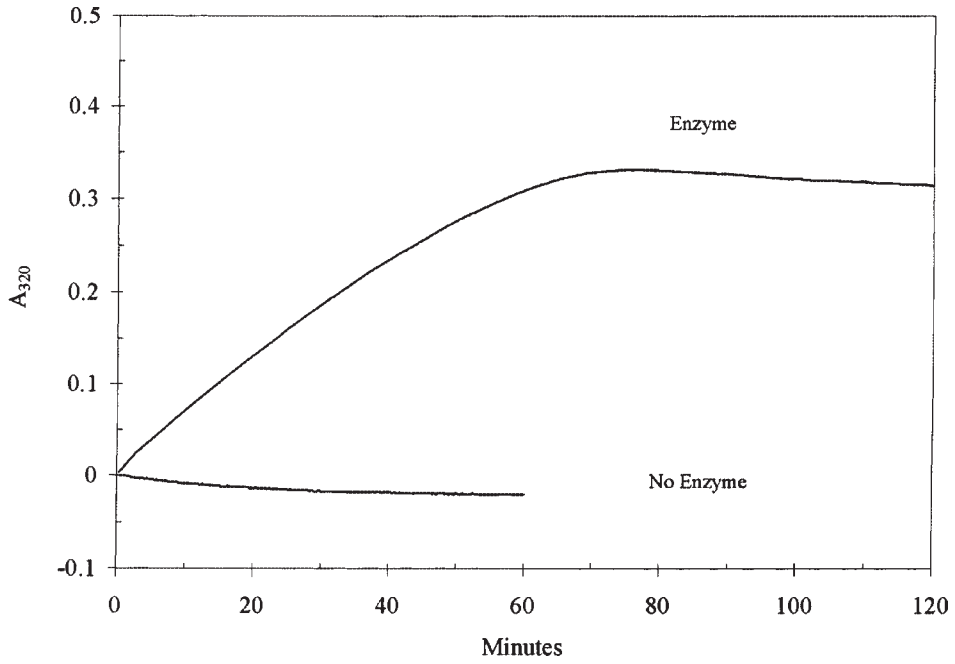

Fig. 1. Spectrophotometric measurement of esterase activity using sodium phosphate buffer. Reaction mixtures prepared as described in Materials and Methods contained 1-naphthyl acetate substrate (50 µM) without and with enzyme protein (40 µg).

Effect of Temperature and pH on Activities of Esterase

The optimum temperature for measurement of diamondback moth esterase activity was in the range of 33–42°C (Fig. 4). No enzyme activity was observed at 5°C or at 60°C at which the enzyme was apparently thermally denatured. The results agreed with that of Kapin and Ahmad (1980) who reported that naphthyl esterase activity of the gypsy moth larvae was apparent up to 50°C, thereafter, dropping sharply at 590 nm.

The optimum esterase activity of the diamondback moth was observed in the pH range from 6.0 to 7.5 (Fig. 5). The results were similar to that of Maa and Chuang (1983) who reported that naphthyl esterase activity of the diamondback moth

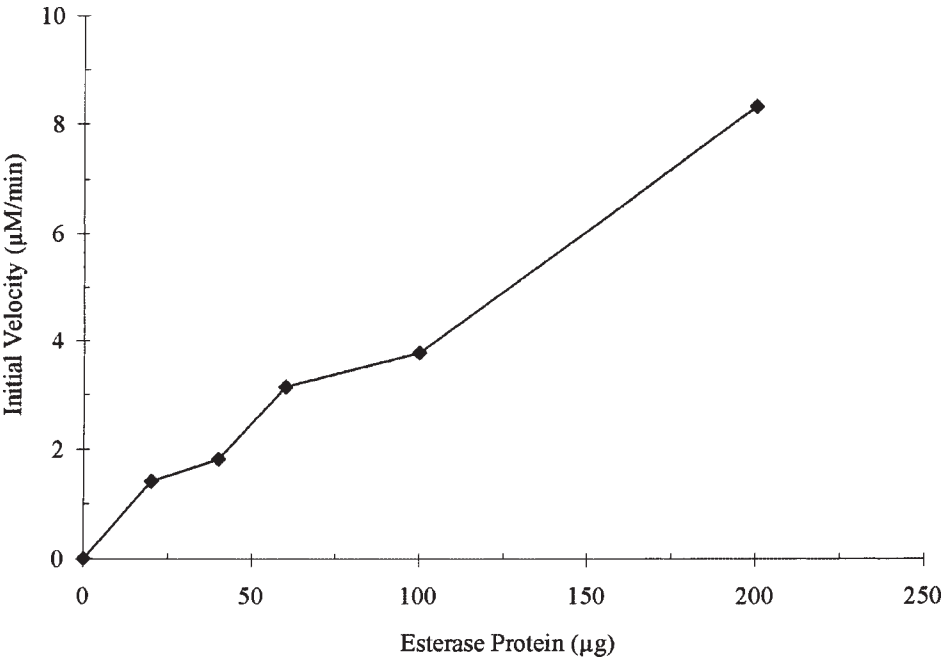

Fig. 2. Effect of esterase protein concentration on the initial velocity of hydrolysis of 1-naphthyl acetate. Reaction mixtures were prepared as described in Materials and Methods with esterase protein concentration from 0 to 200 µg. Initial velocity unit (µM/min) was determined from the increase in A_{320} at the first 10 min.

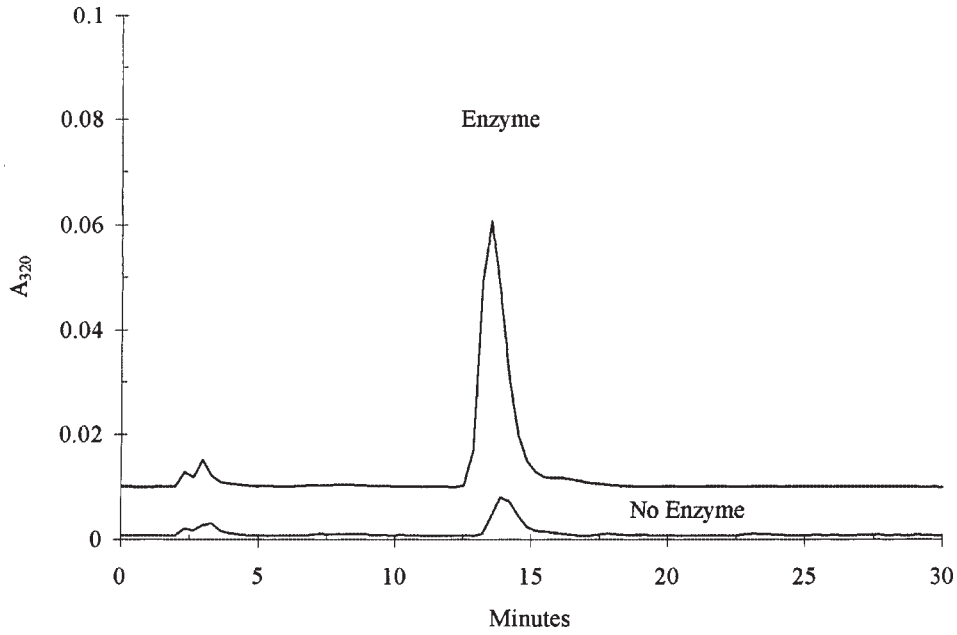

Fig. 3. HPLC spectra of 1-naphthol. After 60 min incubation, 1-naphthyl acetate (400 μM) reacts with or without enzyme (120 μg) at A_{320} nm and 37°C.

larva was observed in the pH range from 6.5 to 7.7 at 600 nm.

Effects of Metal Cations on Activity of Esterase

The effects of metals on the activity of diamondback moth esterase were determined in the presence of various concentrations of Mg^{2+}, Na^{+}, and K^{+}. The results showed that a divalent Mg^{2+} from a range of 5–15 mM enhanced the activity of esterase by 20–25% (Fig. 6). However, monovalent K^{+} and Na^{+} from a range of 50–400 mM inhibited esterase activity by 20% (Fig. 7). These results indicate that diamondback moth esterase activity is only marginally affected by metal ions. For this reason, no salts such as NaCl and KCl were added but 10 mM

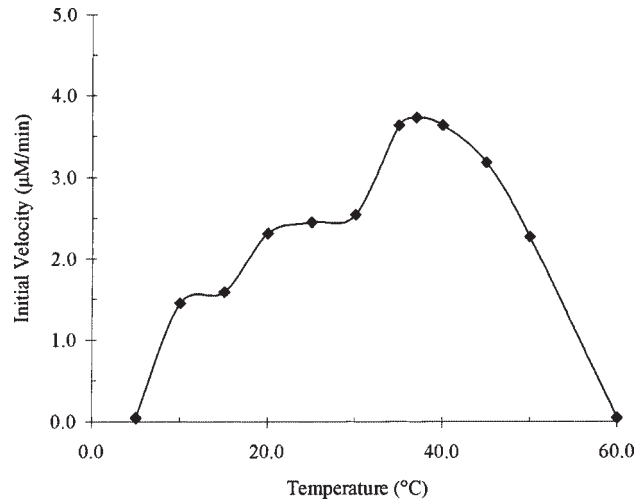

Fig. 4. Effect of temperature on the initial velocity of hydrolysis of 1-nathphyl acetate. Temperature was varied from 5° to 60°C as indicated.

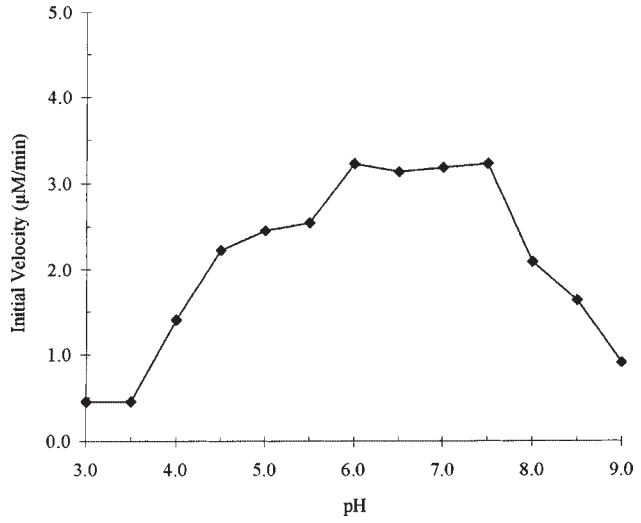

Fig. 5. Effect of pH on the initial velocity of hydrolysis of 1-naphthyl acetate. pH was varied from 3.0 to 9.0 as indicated at 37°C.

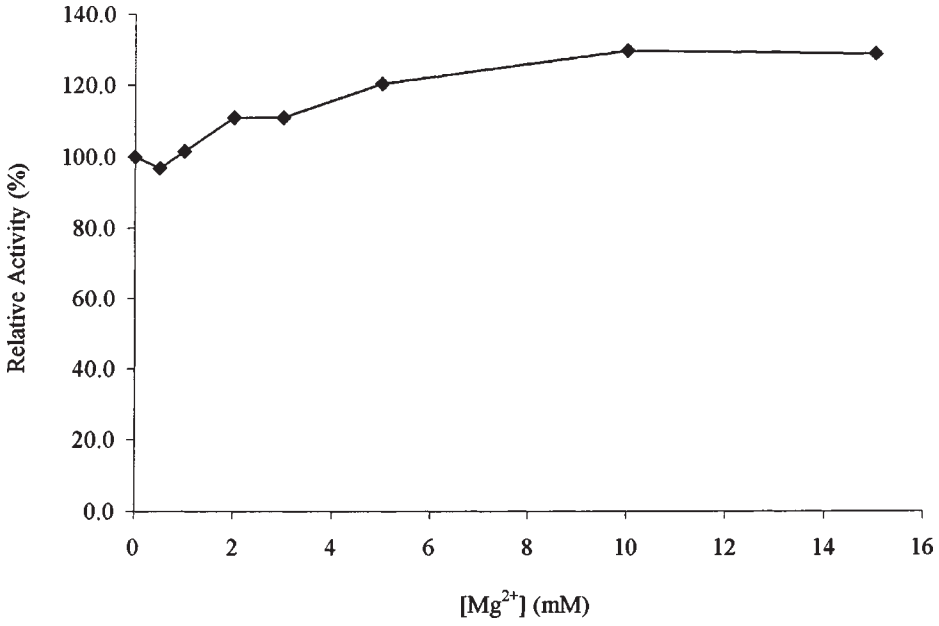

Fig. 6. Effect of Mg^{2+} concentration on the initial velocity of hydrolysis of 1-naphthyl acetate. Mg^{2+} concentration was varied from 0 to 15 mM at 37°C.

$MgCl_2$ was added to the assay buffer. Mastropaolo and Yourno (1981) reported that naphthyl esterase activity was inhibited by 25% by Na^+ ion from a range of 25–300 mM at 235 nm.

Kinetic Parameters Using a 1-Naphthyl Acetate Substrate and an Azadirachtin Inhibitor

The kinetic studies were based on the esterase reaction mechanism for competitive inhibition given by the following reaction scheme (Aldridge and Reinter, 1972):

$$NAc + EH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [EHNAc] \xrightarrow{k_2} EN + HAc \xrightarrow[+H_2O]{k_3} EH + N-OH;$$

$$+I \underset{}{\overset{K_i}{\rightleftharpoons}} EHI \xrightarrow{+NAc} \text{No reaction}$$

Where EH is enzyme, NAc is substrate, 1-naphthyl acetate, I is Inhibitor, EHNAc is enzyme-substrate

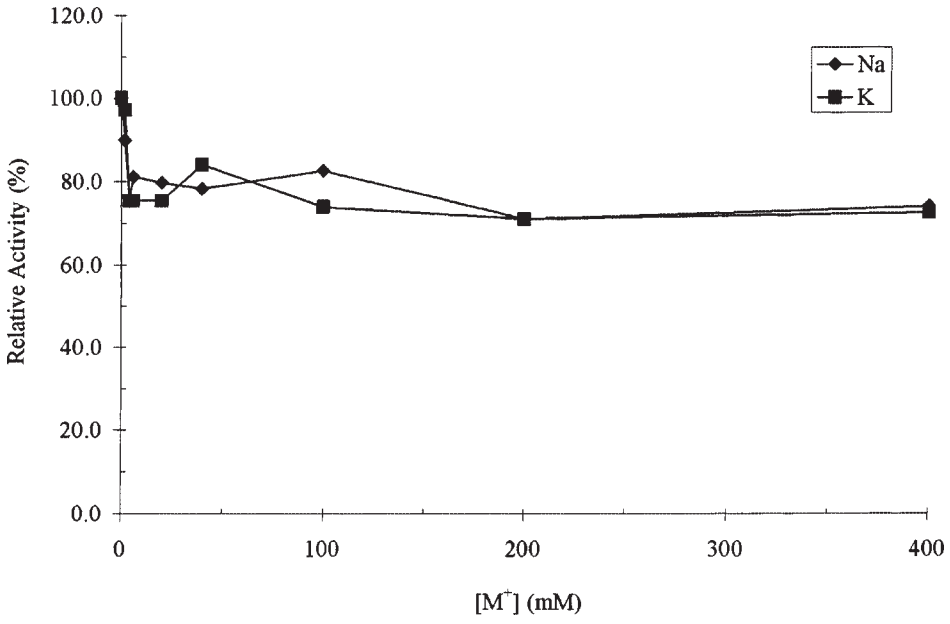

Fig. 7. Effect of Na^+ and K^+ concentration on the initial velocity of hydrolysis of 1-naphthyl acetate. Na^+ and K^+ concentration was varied from 0 to 400 mM at 37°C.

complex, EHI is enzyme-inhibitor complex, HAc is acetate acid, and N-OH is product, 1-naphthol. From which, according to Lineweaver-Burk plot, the reciprocal rate is for no inhibition

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}; \qquad (1)$$

for competitive inhibition is

$$\frac{1}{V_0} = \frac{\alpha K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}, (\alpha = 1 + \frac{[I]}{K_i}). \qquad (2)$$

Where V_0 is the initial enzyme reaction velocity; V_{max} is the maximum enzyme reaction velocity; S is the substrate concentration; I is the inhibitor concentration, K_i is the inhibition constant, and K_m is the Michaelis constant.

The calculations of the kinetic thermodynamic parameters (K_m, V_{max} and K_i) of esterase were done by double-reciprocal plotting $1/V_0$ vs. $1/[S]$ to determine the intercepts and the slopes of the linear transformation (Fig. 8). The Michaelis constant is independent of the enzyme concentration but depends on pH, type of buffer, and temperature in the studies of enzyme kinetics. The values of K_m and V_{max} are 28 ± 1.9 μM and 6.0 ± 0.1 μM/min at 37°C, respectively. The K_m and V_{max} values are similar to the values of 35 ± 4 μM and 6.1 ± 0.2 μM/min, respectively, at pH 7.5 from diamondback moth larval esterase reported by Maa and Chuang (1983). Kapin and Ahmad (1980) also reported that the K_m value was 42.5 μM from gypsy moth (*Lymantria dispar* L.) esterase. Similar K_m and V_{max} values given from the conventional and the new methods indicated that the new method is confirmable.

Azadirachtin is a tertranortrieterpenoid of the limonoid type obtained as a natural product from the neem tree and used as an insecticide. Azadirachtin has three carboxylic esters at C1, C2, and C11 positions. The value of the inhibition constant for azadirachtin is 9 ± 2 μM. Azadirachtin is a reversible competitive inhibitor of esterase using 1-naphthyl acetate as a substrate. It directly binds to the esterase active site therefore reduces the concentration of free enzyme available for naphthyl acetate binding. It possibly allows toxin to stay in diamondback moth for a longer time because of lower activity of detoxifying esterase. Elzen and James (2002) reported that azadirachtin was toxic to *P. xylostella* in a residual insecticide bioassay. Mukherjee and Sharma (1996) reported that azadirachtin induced changes in midgut esterase activity of the final instar larvae of *Spodoptera litura* (F) fed in increasing amount of azadirachtin (30 and 50 ppm).

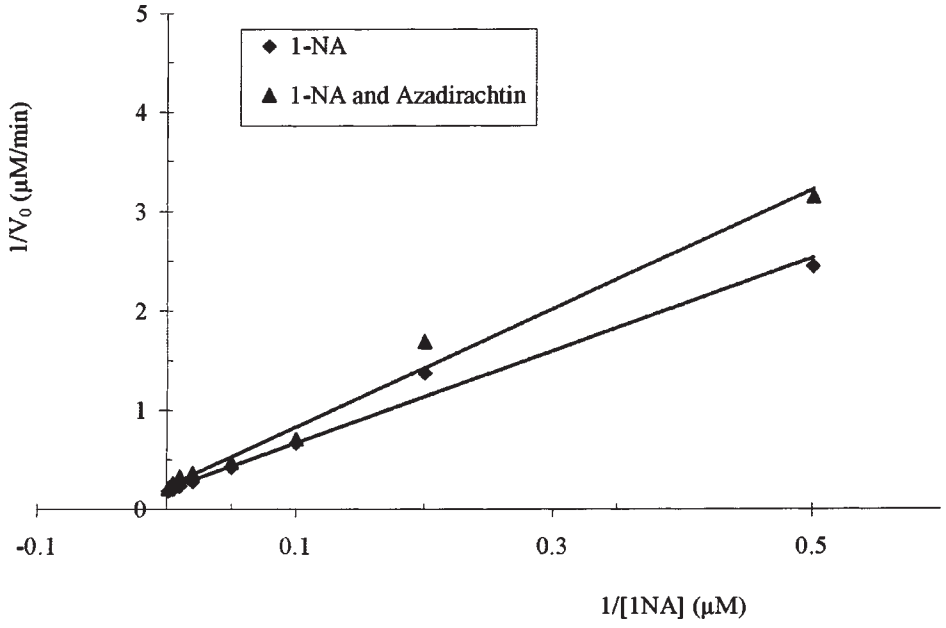

Fig. 8. A double reciprocal (Lineweaver-Burk) plot of 1/initial velocity, V_0, of the competitively inhibited Michaelis-Menten esterase vs. 1/1-naphthyl acetate (μM), in the presence and absence of a competitive inhibitor, azadirachtin. 1-Naphthyl acetate concentrations were varied from 0 to 1,000 μM at 37°C and pH 7.0.

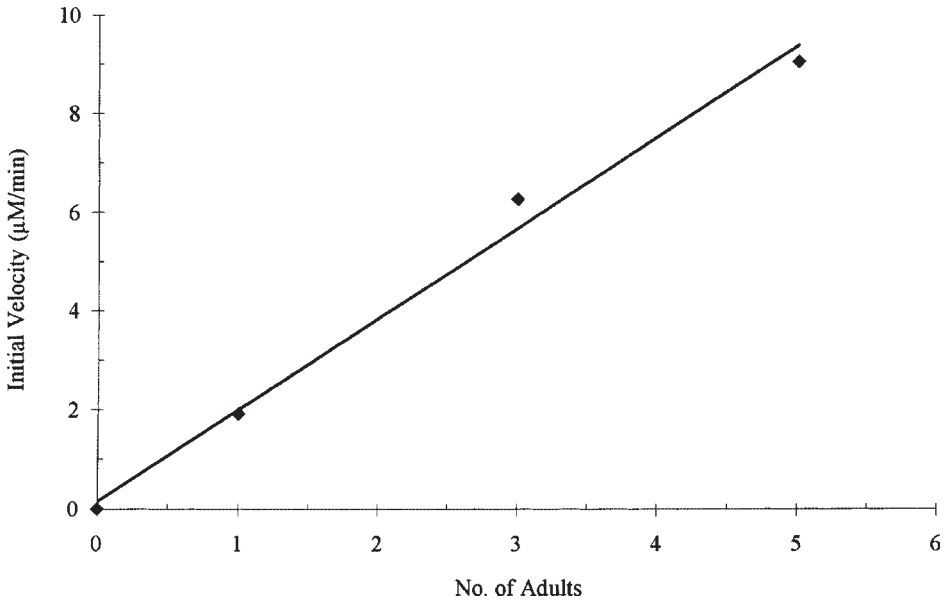

Fig. 9. Effect of number of adults on the initial velocity of hydrolysis of 1-naphthyl acetate. One, three, or five diamondback moths were homogenized in 25 μl of homogenized solution.

Effect of Number of Adults on Activities of Esterase

Extracts of one, three, or five adult diamondback moths were tested for the sensitivity of the method. Increasing the number of adults in the extract predictably increased the initial velocity (Fig. 9). Esterase activity was detectable by this assay in extracts of a single moth. This assay can detect as low as 0.6 μM 1-naphthol at the absorbance signal of 1.5×10^{-3} (O.D.). The assay is suitable for the K_m values at the micromolar level in the kinetic analysis of esterases.

The continuous spectrophotometric assay for esterase activity presented in this work offers improvements over traditional dye-product assays in that it is simple, rapid, accurate, and sensitive. It will be essential in the purification and characterization of esterases from insects. The assay will be fundamental to studies of kinetics and mechanism of esterases. This technique will be useful in pest and pesticide resistance management.

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